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Crystallographic and Fluorescence Studies of the Interaction of Haloalkane Dehalogenase with Halide Ions. Studies with Halide Compounds Reveal a Halide Binding Site in the Active Site[‡]

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ABSTRACT: Haloalkane dehalogenase from *Xanthobacter autotrophicus* GJ10 catalyzes the conversion of 1,2-dichloroethane to 2-chloroethanol and chloride without use of oxygen or cofactors. The active site is situated in an internal cavity, which is accessible from the solvent, even in the crystal. Crystal structures of the dehalogenase enzyme complexed with iodoacetamide, chloroacetamide, iodide, and chloride at pH 6.2 and 8.2 revealed a halide binding site between the ring NH's of two tryptophan residues, Trp-125 and Trp-175, located in the active site. The halide ion lies on the intersection of the planes of the rings of the tryptophans. The binding of iodide and chloride to haloalkane dehalogenase caused a strong decrease in protein fluorescence. The decrease could be fitted to a modified form of the Stern-Volmer equation, indicating the presence of fluorophores of different accessibilities. Halide binding was much stronger at pH 6.0 than at pH 8.2. Assuming ligand binding to Trp-125 and Trp-175 as the sole cause of fluorescence quenching, dissociation constants at pH 6.0 with chloride and iodide were calculated to be 0.49 ± 0.04 and 0.074 ± 0.007 mM, respectively. Detailed structural investigation showed that the halide binding site probably stabilizes the halide product as well as the negatively charged transition state occurring during the formation of the covalent intermediate.

Haloalkane dehalogenase from *Xanthobacter autotrophicus* GJ10 is a globular α/β protein that converts 1-haloalkanes to the corresponding alcohols and a halide ion, without use of oxygen or cofactors (Keuning et al., 1985; Janssen et al., 1989; Franken et al., 1991). Substrates for the enzyme are methyl chloride, ethyl chloride, 1,2-dichloroethane, 1,2-dibromoethane, and other environmental pollutants (Keuning et al., 1985). The three-dimensional structure of the enzyme has been elucidated by Franken et al. (1991) using X-ray crystallography. The catalytic residues were proposed to be Asp-124, His-289, and Asp-260, which are located in an internal cavity. This cavity is about 37 \AA^3 in size ($1 \text{ \AA} = 0.1 \text{ nm}$) and is lined with hydrophobic residues. Even in the crystal the cavity must be reachable from the solvent, because $[\text{Au}(\text{CN})_2]^-$, used as a heavy atom derivative in the structure determination, was found to bind in this cavity after soaking the crystals in a solution of the heavy atom compound (Franken et al., 1991). The proposed reaction mechanism proceeds in two steps (Franken et al., 1991). First, the negatively charged O_δ atom of Asp-124 performs a nucleophilic attack on the C_1 carbon atom of the substrate, resulting in an intermediate ester and a halide ion. The aromatic rings of two tryptophans that point into the cavity could help stabilize the negatively charged transition state as well as the halide ion produced. Subsequently, a hydrolytic water molecule, activated by His-

289, cleaves this intermediate, releasing the alcohol product and restoring the Asp-124 side chain (Figure 1).

Iodoacetamide has been reported to be an inhibitor for the dehalogenase (Keuning et al., 1985). However, the absence of cysteine residues in the putative active site cavity raised the question of what the mechanism of the inhibitory action of iodoacetamide could be. Therefore, we studied the interaction of iodo- and chloroacetamide with the dehalogenase in more detail, both in the crystalline state and in solution. From the experiments we report here, it appears that iodo- and chloroacetamide are poor substrates for the enzyme and that inhibition most probably occurs as a result of the presence of halide ions. The soaking experiments clearly revealed a halide binding site between the ring nitrogens of the two tryptophans located in the active site cavity (Trp-125 and Trp-175).

To corroborate our crystallographic results, we determined the fluorescence of dehalogenase in solution as a function of halide concentration. Iodide is widely used as a strong quenching agent in protein fluorescence studies (Eftink, 1991). Since halide ions quench tryptophanyl fluorescence, it should be possible to determine the binding of halide ions and halogenated substrates in the neighborhood of tryptophan residues by measuring changes in fluorescence. If the two active site cavity tryptophans are involved in halide binding, then a strong quenching effect should be expected. The fluorescence experiments presented here show that this is indeed the case, in complete agreement with the crystallographic results.

MATERIALS AND METHODS

Materials. For the crystallization and soaking experiments, ammonium sulfate, iodoacetamide, chloroacetamide, potassium iodide, and sodium chloride were used without any further purification. For the activity and fluorescence measurements,

[‡] The atomic coordinates of the final models have been deposited with the Brookhaven Protein Data Bank [accession numbers 1EDA, 1EDB, 1EDC, and 1EDD for the iodoacetamide-dehalogenase, the chloroacetamide-dehalogenase, the iodide-dehalogenase, and the chloride-dehalogenase complexes, respectively; Bernstein et al. (1977)].

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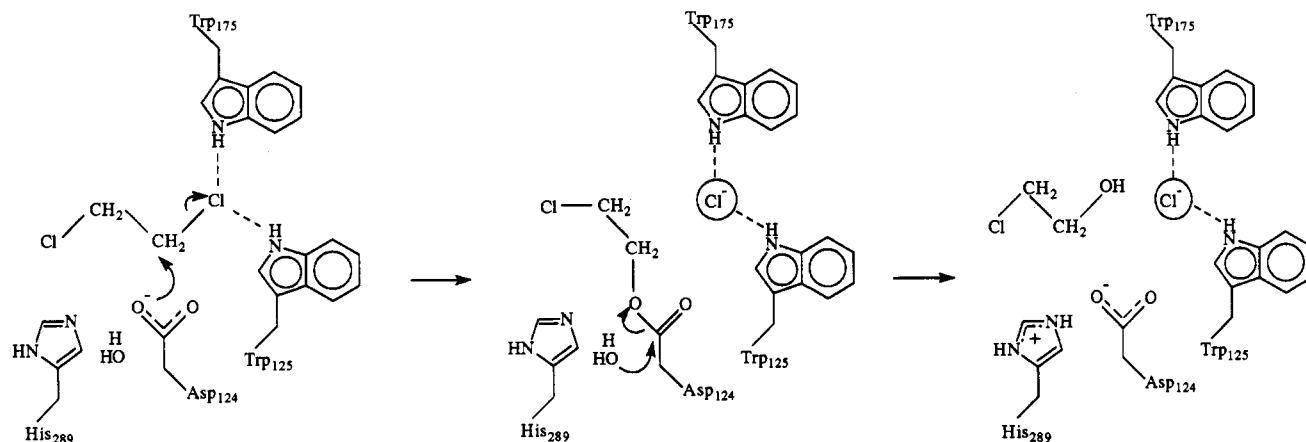


FIGURE 1: Proposed reaction mechanism of haloalkane dehalogenase from *X. autotrophicus* GJ10 (based on Franken et al. (1991) and the results of the present study).

Table I: Data Collection and Stereochemical Quality of the Dehalogenase Complexes

	iodoacetamide-dehalogenase complex	chloroacetamide-dehalogenase complex	iodide-dehalogenase complex	chloride-dehydrogenase complex
pH	6.2	6.2	8.2	8.2
cell dimensions (Å)	$a = 95.1$ $b = 73.0$ $c = 41.4$	$a = 95.4$ $b = 72.9$ $c = 41.4$	$a = 94.9$ $b = 73.1$ $c = 41.5$	$a = 94.4$ $b = 72.5$ $c = 41.3$
resolution range (Å)	7–2.2	7.0–2.0	7.0–2.3	7.0–2.1
total number of observations	34 777	47 248	32 419	44 354
number of unique reflections	12 376	16 306	12 054	15 482
number of discarded observations ^a	530	2841	1152	1645
R_{merge}^b (%)	6.11	4.03	6.65	4.71
completeness of the data (%)	81.2 (∞ –2.19 Å)	84.8 (∞ –2.01 Å)	89.5 (∞ –2.29 Å)	89.2 (∞ –2.10 Å)
Quality of the Final Model				
number of residues	1–310	1–310	1–310	1–310
number of solvent molecules	214	192	130	172
overall B factor (Å ²)	14.3	12.2	11.6	11.8
B factor of the halide ion (Å ²)	13.9	6.0	10.2	1.0
final R factor ^c (%)	16.5	16.9	17.2	16.8
estimated coordinate error ^d (Å)	0.13	0.11	0.13	0.17
rms deviations from ideality for				
bond lengths (Å)	0.009	0.009	0.011	0.011
bond angles (deg)	2.9	2.8	3.2	2.9

^a Intensities which deviated too much (more than 3σ from mean intensities) or were negative. ^b $R_{\text{merge}}(\%) = [\sum_{hkl} \sum_{\text{ref}} |I(hkl, j) - \bar{I}(hkl)|] / [\sum_{hkl} \sum_{\text{ref}} I(hkl, j)] \times 100$. ^c R factor (%) = $[\sum ||F_{\text{obs}}| - |F_{\text{calc}}||] / \sum |F_{\text{obs}}| \times 100$. ^d Derived from a σ_A plot (Read, 1986).

chloroacetamide and iodoacetamide were recrystallized from water to remove contaminating halide.

Crystallization and Soaking Experiments. Purified haloalkane dehalogenase from *X. autotrophicus* GJ10 (Keuning et al., 1985) was crystallized by vapor diffusion techniques as described before (Rozeboom et al., 1988), after an extra purification step on a Mono Q ion-exchange column had been performed.

All soaking experiments were carried out at room temperature. Native dehalogenase crystals were soaked for 2 days in a mother liquor of a 62% saturated (at 0 °C) solution of ammonium sulfate in 100 mM bis-Tris-H₂SO₄ buffer of pH 6.2, containing 5 mM iodoacetamide, 5 mM chloroacetamide, 5 mM potassium iodide, or 10 mM sodium chloride, respectively. For the latter two soaking trials, the pH of the mother liquor was raised to pH 8.2. All soaked crystals were isomorphous with the native dehalogenase crystals (space group $P2_12_12$), and the soaking with the different compounds had a negligible effect on the unit cell dimensions [$a = 94.8$ Å, $b = 72.8$ Å, $c = 41.4$ Å; Franken et al. (1991)] (Table I).

Diffraction Data Collection. All data were collected at room temperature on an Enraf Nonius FAST area detector system (Enraf Nonius, Delft, The Netherlands). The detector

was equipped with a CAD4 κ -goniostat with graphite-monochromatized Cu K α radiation from an Elliot GX21 rotating anode X-ray generator. The MADNES package (Messerschmidt & Pflugrath, 1987) was used for data collection and processing. Profile fitting and local scaling of the data sets were done according to Kabsch (1988). Merging of the data was performed using software from the Groningen BIOMOL crystallographic package. More details of the data processing are shown in Table I.

Crystallographic Refinement. The four dehalogenase complexes were refined using the TNT package (Tronrud et al., 1987). The starting models for all four complexes were the refined 1.9-Å native atomic coordinates of the enzyme at pH 6.2 (Verschuieren et al., 1993a). All solvent molecules were deleted from the starting model to avoid model bias with respect to the halide ions. The TNT suite of programs was adapted, whenever necessary, in order to implement the scattering factors of the iodide ion (Lee, 1969).

Alternating cycles of coordinate and temperature factor refinement and manual rebuilding on a PS390 Evans and Sutherlands graphics system were performed with the programs FRODO (Jones, 1978) and O (Jones et al., 1991), using σ_A weighted (Read, 1986) $(2m|F_{\text{obs}}| - D|F_{\text{calc}}|) \exp(i\alpha_{\text{calc}})$

electron density maps. A halide atom was built in the respective models only when a density peak in an $|F_{\text{obs}}|$ -(complex) - $|F_{\text{obs}}|$ -(native) difference Fourier was consistently higher than 10σ above the background level.

Dehalogenase Assays. Dehalogenase activities were determined as described previously (Keuning et al., 1985). For the determination of kinetic constants, the enzyme was incubated with varying concentrations of substrate and halide ion release was followed using a colorimetric assay (Bergmann & Sanik, 1957).

Fluorescence Measurements. The binding of halide ions was followed by steady-state fluorescence measurements at 25 °C on a SLM Aminco SPF500-C spectrofluorometer. Dehalogenase (1 μM) was dissolved in TEM buffer (50 mM Tris-H₂SO₄ (pH 8.2) containing 1 mM EDTA and 1 mM β -mercaptoethanol) or PEM buffer (50 mM NaH₂PO₄-NaOH (pH 6.0) containing 1 mM EDTA and 1 mM β -mercaptoethanol). The excitation wavelength was 290 nm, and spectra were recorded in the range of 300–500 nm. For quenching experiments, halides were added from stock solutions freshly prepared in the same buffer as the enzyme. The decrease in fluorescence was corrected for dilution, which did not exceed 10%.

Fluorescence quenching data were analyzed with the Stern-Volmer equation (1), which assumes dynamic quenching and describes the ratio of unquenched fluorescence (F_0) to the remaining fluorescence (F) as a function of the concentration of the quenching agent ($[L]$) and the Stern-Volmer quenching constant (K_{SV}) (Eftink, 1991).

$$F_0/F = 1 + K_{\text{SV}}[L] \quad (1)$$

Data were also analyzed with the modified Stern-Volmer equation (2), which describes the effect of quencher concentration on the change of fluorescence in the case of a protein with two classes of fluorophores, of which a fraction f_a is accessible to the quenching agent and is quenched with a Stern-Volmer constant of K_{SV} (Lehrer, 1971).

$$\frac{F_0}{F_0 - F} = \frac{1}{f_a} + \frac{1}{f_a K_{\text{SV}}} \frac{1}{[L]} \quad (2)$$

If quenching is caused by binding of a ligand to a single fluorescent group and the dissociation constant of the ligand and the protein is much larger than the concentration of ligand binding sites, a plot of $F_0/(F_0 - F)$ vs $1/[L]$ can be used to estimate the association constant (K_a) (Ward, 1985). The plot has a slope of $F_0/(\Delta F_{\text{max}} K_a)$ and an intercept of $F_0/\Delta F_{\text{max}}$, in which ΔF_{max} is the difference in fluorescence between the free and the ligand-saturated protein. The plot has the same form as that of eq 2, with K_{SV} corresponding to K_a . The dissociation constants were calculated using a spreadsheet program with linear regression or the ENZFITTER program of Leatherbarrow (1987).

RESULTS

In the course of our studies on the interaction of haloacetamides and halide ions with dehalogenase, four different complexes of the enzyme were solved by X-ray crystallography. Table I lists the pertinent statistics of the crystallographic refinement of these complexes. The final R factors and maximum resolutions for the four complexes were 16.5% (2.2-Å resolution) for the iodoacetamide-dehalogenase complex, 16.9% (2.0 Å) for the chloroacetamide-dehalogenase complex, 17.2% (2.3 Å) for the iodide-dehalogenase complex, and 16.8% (2.1 Å) for the chloride-dehalogenase complex, respectively. The mean positional errors in atomic coordinates

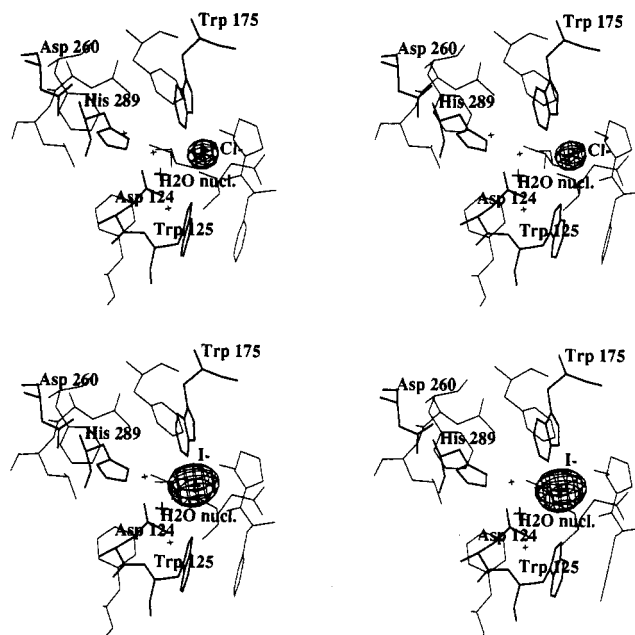


FIGURE 2: (a, top) $|F_{\text{obs}}|$ (chloroacetamide) - $|F_{\text{obs}}|$ (native) difference Fourier, showing the electron density of the chloride ion situated in the active site cavity of the dehalogenase-chloroacetamide complex. The tryptophan residues, the catalytic triad, and the hydrolytic water molecule (H₂O nucl.) are shown with thicker lines. Crosses indicate the positions of water molecules. The $|F_{\text{obs}}|$ (chloride) - $|F_{\text{obs}}|$ (native) difference map is virtually the same. (b, bottom) $|F_{\text{obs}}|$ (iodide) - $|F_{\text{obs}}|$ (native) difference Fourier, showing the electron density of the iodide ion in the active site cavity of the dehalogenase-iodide complex. The $|F_{\text{obs}}|$ (iodoacetamide) - $|F_{\text{obs}}|$ (native) difference map is virtually the same.

for the complexes were estimated from σ_A plots (Read, 1986) to be around 0.15 Å. Further details about the refinement statistics are listed in Table I.

Iodoacetamide-Dehalogenase Complex. Since iodoacetamide was reported to be an inhibitor of the haloalkane dehalogenase (Keuning et al., 1985), we started the soaking experiments with this compound, aiming to elucidate the inhibition mechanism. Soaking native dehalogenase crystals in a 5 mM iodoacetamide solution of pH 6.2 revealed a large spherical electron density located between the two tryptophans (Trp-125 and Trp-175) in the active site cavity. Both an $|F_{\text{obs}}|$ (iodoacetamide) - $|F_{\text{obs}}|$ (native) difference Fourier and a σ_A weighted (Read, 1986) $m|F_{\text{obs}}|$ (iodoacetamide) - $D|F_{\text{calc}}|$ (native) difference map showed one clear single difference peak at the level of over 30σ above background, consistent with an iodide ion. No clear density could be seen for the acetamide moiety of the compound. From these results, we conclude that an iodide ion is bound in the active site. It is firmly stabilized by the side-chain NH's of the two tryptophan aromatic rings (Figure 2b). Table II summarizes the distances of the iodide ion to the neighboring atoms in the cavity. No other clear difference density could be observed, indicating that no other reaction sites for iodoacetamide are present in the dehalogenase molecule. The four cysteine residues in the structure also showed no additional electron density, thereby excluding the earlier assumption that iodoacetamide could inhibit the dehalogenase by modification of one or more cysteines in the enzyme (Keuning et al., 1985).

Chloroacetamide-Dehalogenase Complex. A result very similar to that with the iodoacetamide was obtained by soaking native dehalogenase crystals in a 5 mM chloroacetamide solution at pH 6.2. Also in this case, spherical density for a chloride ion was found in the active site cavity, being the only peak at a level of 11σ in an $|F_{\text{obs}}|$ (chloroacetamide) -

Table II: Distances of the Halide Ion in the Complexes to Its Neighboring Non-Carbon Atoms (≤ 5.0 Å)

atom of dehalogenase	iodoacetamide–dehalogenase distance to I ⁻ (Å)	chloroacetamide–dehalogenase distance to Cl ⁻ (Å)	iodide–dehalogenase distance to I ⁻ (Å)	chloride–dehalogenase distance to Cl ⁻ (Å)
Trp-125 N _{ε1}	3.7	3.4	3.6	3.4
Trp-175 N _{ε1}	3.4	3.2	3.4	3.3
Asp-124 O _{δ1}	4.8		4.6	5.0
Asp-124 O _{δ2}	4.7	4.8	4.6	4.8
Phe-222 O	4.3	3.9	4.2	4.0
Pro-223 N	3.9	3.5	4.0	3.6
H ₂ O	3.4	3.2	3.3	3.2

Table III: Hydrolysis of Haloacetamides by Haloalkane Dehalogenase^a

	K_m (mM)	V_{max} ($\mu\text{mol min}^{-1} \text{mg}^{-1}$)	k_{cat}/K_m ($\text{s}^{-1} \text{M}^{-1}$)
1,2-dichloroethane	0.68 ± 0.07	4.5 ± 0.3	$(3.8 \pm 0.7) \times 10^3$
chloroacetamide ^b	>100	>0.08	
bromoacetamide	63 ± 7	0.83 ± 0.08	7.7 ± 1.6
iodoacetamide	58 ± 7	0.40 ± 0.1	4.0 ± 1.5

^a Dehalogenase assays were performed by measuring halide release at pH 8.2. ^b Chloroacetamide dehalogenase activities increased linearly up to concentrations of 100 mM, above which substrate inhibition occurred.

$|F_{\text{obs}}|(\text{native})$ difference Fourier. This chloride ion was also stabilized between the ring nitrogens of Trp-125 and Trp-175 and was nearly lying in the plane of the two tryptophan side chains (Figure 2a). Likewise, we could not observe any additional density for the acetamide part of the reagent. The distances of the chloride ion to its neighboring atoms are listed in Table II.

Chloride–Dehalogenase and Iodide–Dehalogenase Complexes. To confirm that the halide binding site in the active site cavity in the dehalogenase structure also exists at pH 8.2, the pH at which the enzyme reaches its optimal activity, two other soaking experiments were performed with the native dehalogenase crystal in a 10 mM sodium chloride and a 5 mM potassium iodide solution at this pH. Again, in both complexes a spherical electron density appeared between the two tryptophans on $|F_{\text{obs}}|(\text{chloride}) - |F_{\text{obs}}|(\text{native})$ and $|F_{\text{obs}}|(\text{iodide}) - |F_{\text{obs}}|(\text{native})$ difference Fouriers (Figure 2), which were 24σ and 30σ above background, respectively, indicating that at pH 8.2 also a halide ion can be bound in the active site cavity. The halides are bound at the same position as in the complexes with chloroacetamide and iodoacetamide. The atoms surrounding these chloride and iodide ions with their distances are given in Table II and appear, within the estimated error limit, to be identical to the atoms interacting with the chloride and halide ions from the haloacetamides.

Hydrolysis of Haloacetamides. To investigate whether the halide binding observed in the crystal structures of the dehalogenase soaked in haloacetamides was due to contaminating halide ions present in the haloacetamide solution, or whether the enzyme hydrolyzes the haloacetamides producing the halide ions, we determined the catalytic activity of the enzyme on purified haloacetamides. From Table III, it is clear that dehalogenase catalyzes the release of halide ions from chloroacetamide, bromoacetamide, and iodoacetamide. The activity with chloroacetamide was very low. A K_m value could not be determined since it was above 100 mM, a concentration at which substrate inhibition occurred. The K_m values with bromoacetamide and iodoacetamide were around 60 mM, which is much higher than with 1,2-dichloroethane, and the V_{max} was lower. As a result, the specificity constant (k_{cat}/K_m) of the enzyme for haloacetamides was 500–1000-fold lower than for 1,2-dichloroethane.

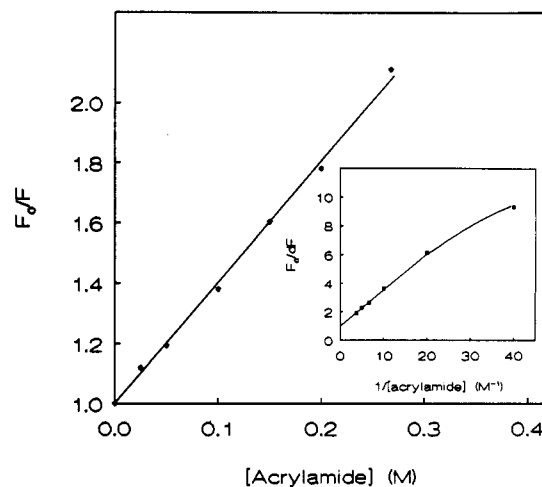


FIGURE 3: Quenching of haloalkane dehalogenase fluorescence by acrylamide. Measurements were done at pH 8.2 in 50 mM Tris-H₂SO₄ buffer. The fluorescence is plotted as the reciprocal of the fraction of unquenched fluorescence (eq 1, Stern–Volmer plot). The inset shows the modified Stern–Volmer plot (eq 2).

Fluorescence Quenching. Haloalkane dehalogenase contains six tryptophan residues, of which two are located in the active site cavity. Using fluorescence measurements, we have studied the binding of halide ions to the enzyme. When dissolved in 50 mM Tris buffer at pH 8.2, the dehalogenase showed an emission maximum at 346.5 nm, which is typical of tryptophanyl fluorescence. The quenching of dehalogenase fluorescence caused by acrylamide followed the simple Stern–Volmer relationship (eq 1, Figure 3). Acrylamide is a polar nonionic quencher that has access to all but the most buried residues. The linear increase of F_0/F with increasing acrylamide concentrations and the value of the Stern–Volmer constant K_{SV} indicate that all six tryptophan residues are relatively well accessible to acrylamide (Eftink & Ghiron, 1976).

Addition of iodide at different concentrations to a solution of dehalogenase in Tris buffer at pH 8.2 caused a decrease in fluorescence (Figure 4). As with acrylamide, there was no shift of the emission maximum, indicating that the hydrophobicity of the environment of the fluorescent groups did not change. Iodide is a large polar anion considered to have access only to surface tryptophans. The quenching data obtained with a range of iodide concentrations at pH 8.2 (Tris buffer) or pH 6.0 (phosphate buffer) (Figure 5) could not be fitted with the Stern–Volmer equation (1). The modified Stern–Volmer equation (2) did fit the experimental data (Figure 5, insets), indicating the presence of fluorophores of different accessibility to iodide ions. The fractional accessibility of 0.5 calculated from these plots is higher than expected if only the fluorescence of the two cavity-exposed tryptophan residues would be quenched.

Chloride ions caused a similar quenching of fluorescence, although higher concentrations were needed. Again, data

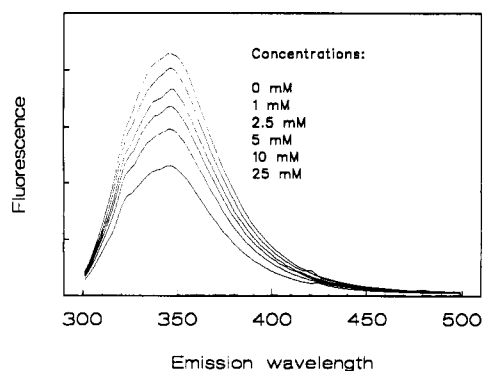


FIGURE 4: Effect of iodide on the steady-state fluorescence emission spectrum of haloalkane dehalogenase. Spectra were recorded at pH 8.2 in TEM buffer as described under Materials and Methods.

could only be fitted to the modified Stern–Volmer equation (2), which gave a fractional accessibility of 0.34 and a higher quenching constant at pH 6.0 than at pH 8.2 (Figure 5). The f_a values obtained with chloride are in agreement with two of the six tryptophans being quenched.

If it is assumed that quenching is caused only by binding of halide ions to the active site tryptophans, then the Stern–Volmer constant in eq 2 corresponds to an association constant between ligand and fluorophore (Ward, 1985). The dissociation constants (K_d) (Table IV) show that iodide binds stronger than chloride and that binding at pH 6 is stronger than at pH 8.2.

DISCUSSION AND CONCLUSION

Conversion of Haloacetamides. To investigate the previously reported inhibitory action of iodoacetamide on the activity of haloalkane dehalogenase (Keuning et al., 1985), we elucidated the crystal structures of the enzyme complexed with haloacetamides. The soaking experiments with iodoacetamide and chloroacetamide at pH 6.2 did not cause significant structural changes in the protein molecule. The residues surrounding the active site retain the native conformation and are not disrupted upon binding the compound. This rules out a conformational change as a possible cause for the reported inhibition by iodoacetamide. The four cysteine residues in the enzyme structure are also not affected. We could not observe modified electron densities of these residues upon binding. Moreover, the nearest cysteine (Cys-150) is 8.5 Å away from the putative active site cavity and is shielded from the cavity by the side chain of Phe-128 (Franken et al., 1991).

The soaking results, however, clearly indicated the binding position of a halide ion, one of the products of the reaction catalyzed by the haloalkane dehalogenase. The halide ion is firmly stabilized in the internal active site cavity between two tryptophan aromatic rings (Trp-125 and Trp-175) which point with their side chains into the cavity. Kinetic results indicated that purified haloacetamides act more as poor substrates than as inhibitors. They are converted at a low rate by the purified dehalogenase. Since dehalogenase in the crystalline state is active (Verschuere et al., 1993b), this could account for the presence of the halide ion in the active site cavity, the more so because the soaking and data collection were done over a period of 4 days. Nevertheless, we cannot rule out the alternative explanation that the presence of a halide ion in the active site cavity is due to contamination of the soaking solution by these ions. Differences in substrate purity or the lower pH used in previous assays might also explain the previously reported inhibitory effect of iodoacetamide on the activity of

the enzyme (Keuning et al., 1985). At pH 7.5, halide ions that contaminate the chemically labile substrate are bound stronger and thus will be more efficient inhibitors than at pH 8.2 (see below).

Binding Site for Halides between the Two Tryptophans of the Active Site Cavity. The crystallographic results obtained from the soaking experiments of haloalkane dehalogenase crystals with halides clearly indicated a halide binding site between the two tryptophan residues (Trp-125 and Trp-175) in the active site cavity. The fluorescence quenching data are in agreement with these results. The cavity contains two of the six tryptophan residues present in the enzyme. Proteins are considered to be poorly penetrated by the negatively charged halide ions, allowing only quenching of the fluorescence of solvent-exposed groups by a collision mechanism. The fractional accessibility (f_a) values obtained for chloride at pH and 6.0 and 8.2 indicate that only two tryptophans are accessible, most probably the ones in the active site. The iodide ion quenching data suggest that three out of six tryptophan residues are accessible. Analysis of the position of the tryptophans in the three-dimensional structure of the dehalogenase shows that Trp-194 is also somewhat accessible. Alternatively, the higher apparent accessibility to iodide could indicate that the two tryptophan residues in the cavity make a greater contribution to the total protein fluorescence than the expected 33%, in which case chloride would not completely abolish fluorescence.

Assuming halide binding as the cause of fluorescence quenching, this change of fluorescence can be used to determine dissociation constants and stoichiometries of ligand binding (Ward, 1985). At pH 6.0 the dissociation constants for halide ions are much lower than at pH 8.2. The most likely explanation for this observation is that in native dehalogenase, without halides bound, the active site His-289 is positively charged at pH 6.0, but is neutral at pH 8.2 (Verschuere et al., 1993a). Since the halide ion in the active site binds in the neighborhood of His-289 and of the negatively charged Asp-124 side chain, the binding at low pH with a positive charge on the His-289 side chain will be more favorable than at high pH.

Iodide ions are bound more strongly than chloride ions. Likewise, the K_m for iodoacetamide is lower than that for chloroacetamide. Although both compounds are poor substrates, it thus appears that interactions between the halogen and the tryptophans in the active site contribute considerably to substrate binding.

Mode of Binding of the Halide Ion between the Tryptophans. In the crystal structure no obvious positively charged group is found in the vicinity of the negatively charged halide ion. The halide ion is stabilized in the nonpolar, hydrophobic environment of the cavity. From the earlier studies of Swaney and Klotz (1970) performed on serum albumin it was already proposed that a cluster of nonpolar residues including a tryptophan residue can offer a very favorable environment for binding small anions. The hydrogen atoms bound to the ring nitrogens of the two tryptophans, Trp-125 and Trp-175, in the cavity of dehalogenase have a slightly positive charge which facilitates positioning of the negatively charged halide ion between the two nitrogen atoms. In addition, the bound anion may be stabilized by nonpolar interactions in the cavity. Whereas positively charged groups are often stabilized through van der Waals contact with the π -electron cloud of aromatic rings and are therefore located above or below the ring (Tüchsen & Woodward, 1987; Burley & Petsko, 1988; Kooystra et al., 1988; Perutz et al., 1986), negatively charged

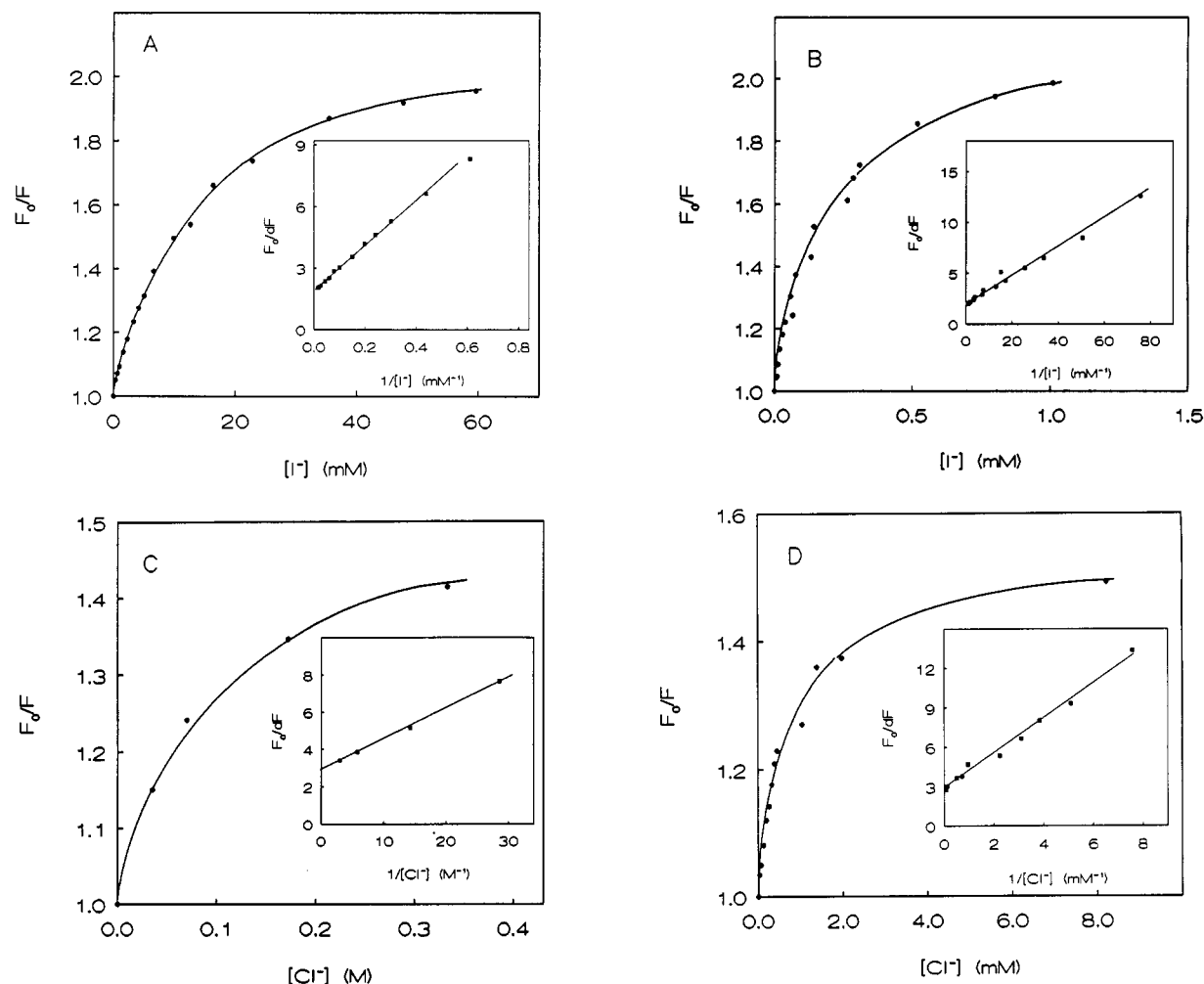


FIGURE 5: Effect of halide ions on the fluorescence of haloalkane dehalogenase: (A) iodide, pH 8.2; (B) iodide, pH 6.0; (C) chloride, pH 8.2; (D) chloride, pH 6.0. Fluorescence was measured in Tris buffer (pH 8.2) or phosphate buffer (pH 6.0) as described in the Materials and Methods. Stern-Volmer plots are shown (eq 1), with plots of the modified Stern-volmer equation in the insets (eq 2).

groups are preferentially located in the plane of the aromatic ring, interacting with the somewhat positively charged aromatic hydrogen atoms. Thomas et al. (1982) have presented such an electrostatic interaction between a negatively charged oxygen and the positively charged aromatic ring hydrogens of a phenylalanine side chain, accompanied by a repulsion of the oxygen by the electron-ring π -cloud of the aromatic ring. This has also been observed for the sulfur-aromatic interactions in proteins (Reid, 1985), where, of 36 proteins examined, almost half of the electronegative sulfur atoms of the cysteine and methionine residues exhibited affinity toward the edge of the planar aromatic rings. The occurrence of these electrostatic interactions between residues in proteins suggests that interactions of polar groups with aromatic rings contribute to protein stability (Burley & Petsko, 1988; Reid, 1985).

The results obtained from the soaking experiments with halides and halide compounds strongly suggest that haloalkane dehalogenase uses the same kind of electrostatic stabilization for the interaction of the side chains of the two tryptophans in the active site cavity, Trp-125 and Trp-175, with the negatively charged halide product. Moreover, the aromatic rings of these two tryptophans are positioned in such a way that they even may contribute to the stabilization of the putative negatively charged transition state in the first step of the reaction pathway of the dehalogenase. The transition state for this step has been proposed to be a pentacoordinated

Table IV: Quenching Constants (K_{SV}), Fractional Accessibilities (f_a), and Dissociation Constants (K_d) of Halides

quenching agent	pH	f_a	K_{SV}^a (M^{-1})	K_d (mM)
acrylamide	8.2	1.0 ± 0.1	4.2 ± 0.2	
chloride	6.0	0.34 ± 0.01	$(2.1 \pm 0.2) \times 10^3$	0.49 ± 0.04
iodide	6.0	0.49 ± 0.01	$(14.8 \pm 1.4) \times 10^3$	0.074 ± 0.007
chloride	8.2	0.35 ± 0.01	17 ± 0.1	57 ± 3
iodide	8.2	0.49 ± 0.01	202 ± 12	5.0 ± 0.3

^a Quenching constants were calculated from the Stern-Volmer equation (acrylamide, eq 1, Figure 3) or the modified Stern-Volmer equation (halides, eq 2, Figure 5), using least-squares fits.

carbon atom with a negative charge developing on the halogen atom that is covalently bound to this carbon (Franken et al., 1991). The interaction of the halogen atom with the tryptophans might lower the transition-state energy and thus facilitate the formation of a covalently bound intermediate (Figure 1).

Site-directed mutagenesis experiments on Trp-125 and Trp-175 in the active site cavity could add extra information on the role of these tryptophans in the reaction mechanism of haloalkane dehalogenase.

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